

Genetically Engineered Rice with Enhanced Iron and Carotenoids

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Abstract

Micronutrient deficiency is one of the major nutritional problem in the population of developing countries. Rice, a staple food contains very low amount of minerals and vitamins in the consumable milled seeds. In mature rice seed, external aleurone layer and embryo accumulates maximum amount of minerals, which are removed during milling leaving behind very low nutrient content in the edible seeds. Therefore, to increase the nutritional components in consumable rice grains, biotechnological strategy have been employed. Rice ferritin gene (Osfer2) has been cloned and overexpressed in Pusa Sugandhi II under the control of the endosperm specific promoter OsglutelinA2. The resulting transgenic milled seeds of Pusa sugandhi II showed up to 7.8 fold of ferritin overexpression and 2.09 fold of iron increment. In addition, phytic acid functions as antinutrient by chelating divalent metals such as iron, zinc that reduces mineral nutrition in cereal grains. The biosynthesis of phytic acid involves a complex metabolic pathway where myo-inositol phosphate synthase (MIPS) and inositol phosphate kinase-1 (IPK1) play the vital role. Aleurone layer specific downregulation of phytic acid biosynthesis through RNAi mediated silencing of mips and ipk gene expression has been employed for indirect enrichment of mineral in transgenic rice seed endosperms. Moreover, it has been noted that the transgenic plants possess no significant differences in morphological as well as other agronomic characters with those of non-transgenic plants. For accumulation of carotenoids (pro-vitaminA) in rice endosperm, phytoene synthase (psy) gene cloned from rice variety has been used together with phytoene desaturase to transform rice variety where both the genes were driven by endosperm-specific glutalin promoter. We are aiming to develop low phytate, low lipoxygenase rice with enhanced iron, zinc and β -carotene enriched nutrition rice.

Keywords: Rice, iron, phytic acid, pro-vitaminA, ferritin, transgenic

Introduction

A large portion of the world population is chronically undernourished and suffers from malnutrition due to micronutrient deficiency. Malnutrition due to deficiency of micronutrients is becoming a severe problem in the world. Under this context, rice as a food crop holds immense global importance as a target because it is the primary food for half of the population in the world, providing more calories than any other single food. It represents the major energy source of millions of people of developing countries. The bran layers of rice contain minerals, vitamins and many other compounds with known health benefits that the polished rice grains do not have.

Iron and carotenoids deficiency are major nutritional problems especially in developing countries. The World Health Organization estimates that iron deficiency anemia (IDA) affects 88% of pregnant and 74% of non-

pregnant women other than children. In India, more than 15% of pre-school children and 20% of the pregnant women suffer from vitamin A deficiency (VAD) that even causes permanent night blindness (West 2002). Biofortified rice with high iron, zinc and pro-vitaminA may play a crucial role in reducing the percentage of malnutrition due to iron, zinc and vitamin A deficiency.

Fe molecules are transported to the storage organs like seeds through different types of transporter protein family members like YSLs (Yellow Stripe Like) and IRT (Iron Related Transporter) that play the crucial role in iron transport mainly into the developing floral parts and the storage tissues like seeds (Kim and Guerinot 2007). The major iron accumulator protein like ferritin is localized in the stroma of chloroplast. It is made up of 24 homologous subunits. Mature ferritin subunit (mol wt.28 kda) accumulates about 4500 iron molecules in its central core as well as releases Fe molecules in a

controlled fashion (Masuda et al 2010). Iron is very low in polished rice as it is accumulated more in the outer layer of grain which is removed during polishing. Overexpression of the ferritin protein in rice seed endosperm as well as the different types of transporter proteins in tissue specific manner will allow storing more bioavailable iron molecules in the polished grain. Biofortified high iron rice could play a positive role in reducing iron deficiency and the percentage of malnutrition due to iron (Datta and Khush 2002).

In order to improve bioavailability of iron and phosphorus from rice, phytic acid content in rice seeds plays an important role. Phytic acid known as *myo*-inositol-1,2,3,4,5,6 hexakisphosphate) or InsP_6 , is the most abundant phosphorus-containing compound in mature seeds, typically representing from 65 to 80% of the mature seed's total phosphorus (P). Cereal and legume seeds contain plenty of phosphorus mostly in the form of phytic acid. Non-ruminants cannot digest this phytic acid efficiently due to lack of phytase enzyme, which is required to remove phosphate from the inositol in the phytate molecule (Kuwano et al 2006). Phytic acid strongly binds to mineral cations like Fe^{2+} , Zn^{2+} , Ca^{2+} and Mg^{2+} to form a mixed salt called phytate. The phytic acid content of grain based diet may contribute to iron and zinc deficiencies (Raboy 2007). To increase mineral (Fe^{2+} , Zn^{2+} etc.) as well as phosphorus bioavailability, a reduction of phytic acid levels in rice seeds is required

Carotenoids, a broad class of 40-carbon isoprenoid of naturally occurring pigments is another essential nutritional parameter which serves mainly as an antioxidant. β -carotene, one of the major antioxidant is the precursor of retinoids, retinol, vitamin A and retinoic acid. As vertebrates cannot synthesize the carotenoids *de novo*, carotenoids enriched polished rice grains may offer a sustainable avenue for improving human nutrition and health. β -carotene is synthesized from the precursor GGPP (geranyl geranyl pyrophosphate) through different types of metabolic enzymes mainly *psy* (phytoene synthase), *crt* (phytoene desaturase) and lycopene cyclase. Endosperm specific overexpression of the genes expressing these enzymes is a promising approach to increase the β -carotene in rice seed endosperm.

Since rice grains are stored mostly before consumption, rice grain quality especially nutritional quality deterioration during storage is a major concern. Lipoxygenases (LOXs) activity in rice grain catalyzes addition of molecular oxygen to polyunsaturated fatty acids to produce an unsaturated fatty acid hydroperoxide. Leenhart et al 2006, has shown that wheat lipoxygenase activity induces greater loss of

carotenoid than loss of vitamin E during bread making. Same problem was reported earlier in case of soybean that the variety with high lipoxygenase activity, produced a high level of TBA and carbonyl (Dahuja and Madaan 2003).

Review of recent advances

Improvement of Iron content in Rice

Iron accumulation and enhanced growth in transgenic lettuce plants expressing iron-binding protein ferritin was reported by Goto et al (1999). Lucca et al (2001) applied genetic engineering approach to improve the level of iron in rice grain. Expression of soybean *ferritin* gene, driven by the endosperm-specific glutelin promoter, leading to higher iron and zinc levels in transgenic rice was demonstrated by Vasconcelos et al (2003). The nutritional composition of grains of high iron transgenic ferritin rice has been estimated and were found to be comparable to that of non-transgenic counterpart and well within the acceptable range of reported values (Gayen et al 2012)

Upregulation of transporter like ferric reductase gene, *frel* under the root specific *irt1* promoter enhances the iron uptake in rice plants (Ishimaru et al 2007) and overexpression of *irt1* also led to high iron accumulation in the grains (Lee and An 2009). Overexpression of transcriptional factors like *OsIRO2* under *35S* promoter enhanced the uptake of Fe molecules from the soil and helped to transport more iron into the grain (Ogo et al 2007). Overexpression of *nas* in the rice plants through incorporating of *Hvnas1* gene under *35S* or rice *actin1* promoter increased iron content in the seeds (Masuda et al 2009). Transgenic rice plants harboring both *Atnas1* gene under *35S* promoter and *Phaseolus ferritin* gene under endosperm specific *globulin* promoter, gave five to six folds increased iron accumulation in the grain (Wirth et al 2009). Constitutive and tissue specific overexpression of *Osnas* gene also plays the crucial role for development of high iron rice (Zheng et al 2010; Johnson et al 2011).

Loading of carotenoids in rice seed

In the past decades significant progress has been made in the genetic engineering of carotenoid biosynthesis in plants through a combination of molecular, genetic, biochemical and genomic approaches, a nearly complete set of the genes and enzymes that are involved in carotenoid biosynthesis has been characterized. The first engineering of metabolic pathway for biosynthesis of β -carotene (1.6 $\mu\text{g/g}$ total carotenoids) in the endosperm of *japonica* type rice cultivar (Taipei 309) was demonstrated (Ye et al 2000). Later it was demonstrated in *indica* type cultivars designated as "Golden indica rice" (Datta et al 2003) with increased total carotenoids

up to 9.346 $\mu\text{g/g}$ and β -carotene level alone reached up to 3.96 $\mu\text{g/g}$ in polished grains (Datta et al 2006). Successful attempts were also made to generate marker free high carotenoid transgenic lines of *indica* rice that accumulates carotenoids in seed endosperm (Parkhi et al 2005).

Further, in Golden Rice 2 (reported by Syngenta group) ectopic expression of genes involved in carotenoid biosynthetic pathway results in the accumulation of β -carotene up to 31 $\mu\text{g/g}$ in rice endosperm (Paine et al 2005). Functional expression of *psy* from daffodil and maize has been studied and established in golden rice GR1 and GR2, respectively. However carotenoids level of GR 2 degrades during storage and no stable transformation with rice *psy* has been reported yet.

The “*Or*” or “cauliflower orange mutant gene” arose from a spontaneous mutation of a single gene, represents a novel gene mutation in conferring carotenoid accumulation (Lu et al 2006). Identification of potential rate limiting steps in carotenoid biosynthetic pathway and maximal increase of their catalytic activities are considered to have profound effect on manipulation of the total levels of carotenoid accumulation. The *Or* gene mutation exerts its strong effect on tissue containing large number of dividing cells and results in tissue-specific carotenoid accumulation.

Studies Underway

High iron rice

To generate rice seeds with high iron content in the edible endosperm, we are emphasizing on overexpression of endogenous *ferritin* gene cloned from rice variety. To avoid the constitutive expression of the gene, preference has been given on the expression in a tissue specific manner.

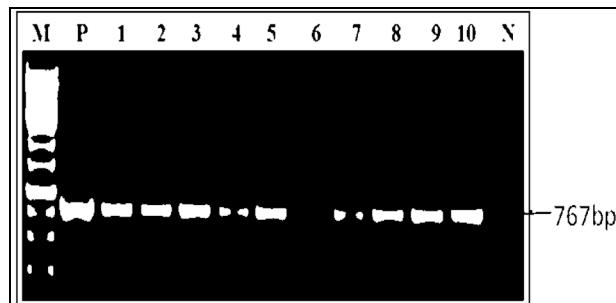


Figure 1. Screening of transgenics with *Osfer2* gene specific PCR. The figure shows the presence of *Osfer2* gene in 10 transgenic T_2 plants. (M= 1 Kb gene ruler, P= positive control, N= negative control, lane 1-10= plants positive for *Osfer2* gene)

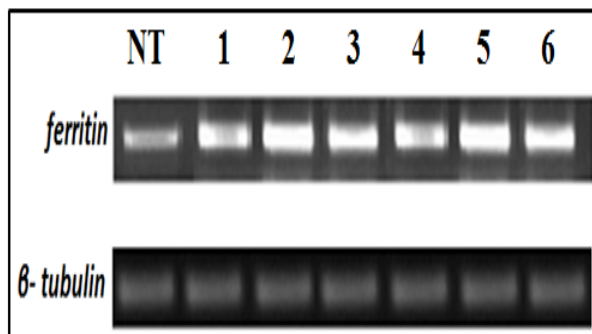


Figure 2. Semi-quantitative RT-PCR of *ferritin* gene from the T_3 transgenic seeds. The figure shows overexpression of *ferritin* gene *Osfer2* in lines 1-6 (276-1-2, 276-1-11, 254-8-7, 150-10-1, 150-10-3 and 150-10-8) as compared to the non-transgenic control (NT). β -tubulin used as reference gene shows similar expression in both non-transgenic and transgenic seeds.

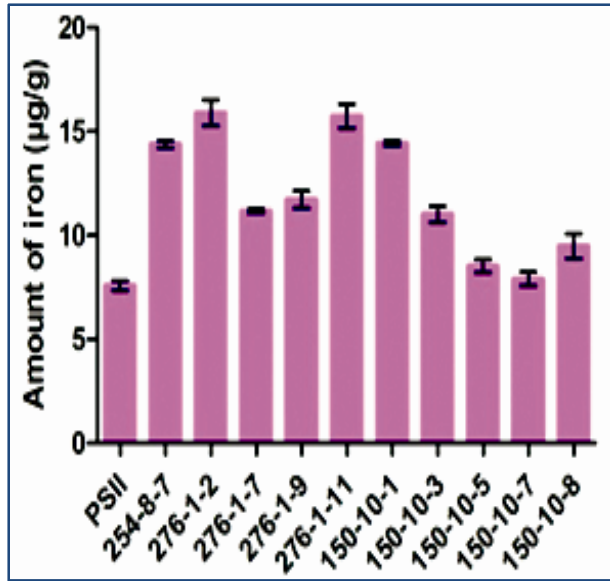
Development of transgenic rice overexpressing the rice ferritin gene

The *ferritin* gene has been cloned from local rice cultivar, Swarna and overexpressed under the control of endosperm specific *GlutelinA2* promoter. The overexpression vector was transformed into *indica* rice cultivar Pusa Sugandhi II and the resulting putative hygromycin resistant transgenic plants were screened through PCR, exhibiting the integration of *Osfer2* gene in transgenic plants (Fig.1). The gene expression, analyzed by semiquantitative PCR, suggests the overexpression of *ferritin* gene in T_3 transgenic seeds (Fig.2).

In RT-PCR analysis, similar level of expression of the housekeeping gene (β -tubulin) in those transgenic seeds indicates single overexpression of the integrated rice *ferritin* gene in the tissue specific manner.

Metal concentration analysis

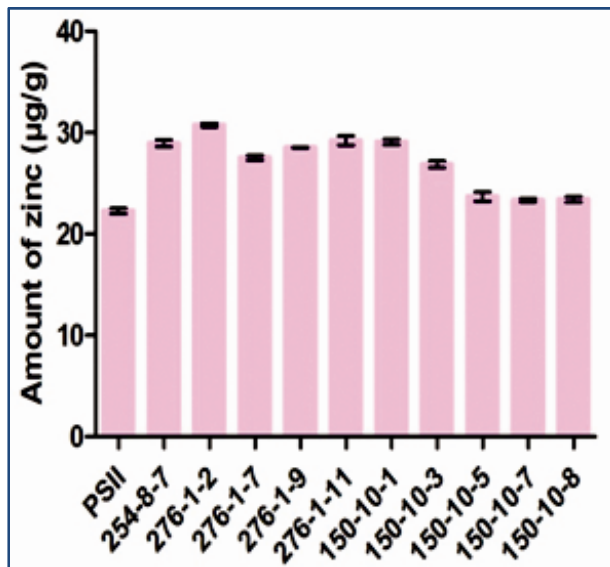
Iron and zinc accumulation was estimated from T_3 milled seeds through Atomic Absorption Spectroscopy (AAS). A wide range of iron (~8.0 to 16.0 $\mu\text{g/g}$ of seed dry weight) and zinc (~23.0 to 30.0 $\mu\text{g/g}$ of seed dry weight) enrichment was observed in the different T_2 transgenic progenies (Fig 3). In comparison to non-transgenic Pusa-sugandhi II, the amount of iron were enhanced from 1.1 to 2.1 fold in the milled seeds of the transgenic plants along with the increment of zinc molecules from 1.04 to 1.36 fold, which was not directly parallel to the iron accumulation level. In 150-10-5, 150-10-7 and 150-10-8, very insignificant increment of iron and zinc molecules was observed ($P > 0.05$).



Among all the T₂ transgenic plants, 276-1-2 and 276-1-11 emerged as maximum iron accumulator due to their ~2 fold iron loading capacities over non-transgenic seeds.

Histochemical analysis of transgenic seeds

Distribution of iron and zinc in the transgenic seeds was confirmed by Pearls Prussian blue and dithizone staining methods respectively (Pearse et al 1972; Ozturk et al 2006). Insoluble blue coloration reflected the distribution of iron (mainly Fe³⁺) in the endosperm along with the aleurone layer and embryo of transgenic seed. However, lighter colour was detected in the endosperm of non-transgenic seeds. For zinc, transgenic seeds had more intense yellowish red coloration towards the centre of the endosperm as compared to non-transgenic seeds, establishing the higher accumulation of zinc molecules particularly in the endosperm.



Similar morphological traits were observed among all the T₂ and T₃ progenies, indicating that no genetic redundancy or seed-iron overload had occurred. Overexpression of *ferritin* in seeds did not hamper the iron homeostasis owing to the large iron sequestration properties of *ferritin* and plants reproduced with normal phenotypic characters.

Low phytate rice

In order to develop low phytate rice, we have cloned the rice *mips* and *ipk1* genes involved in the phytic acid biosynthetic pathway separately under the control of rice aleurone tissue specific promoters respectively in downregulation vectors and transformed into the *indica* rice cultivar, Pusa Sugandhi 2. The molecular and biochemical analyses were performed with the resulting transgenic rice plants confirmed that the transgenic rice seeds had reduced levels of phytate as compared to the non-transgenic control plants (Fig. 4-5).

Figure 3. Amount of iron (µg/g) and zinc (µg/g) in milled T₃ transgenic seeds as compared to non-transgenic control (Pusa Sugandhi II)

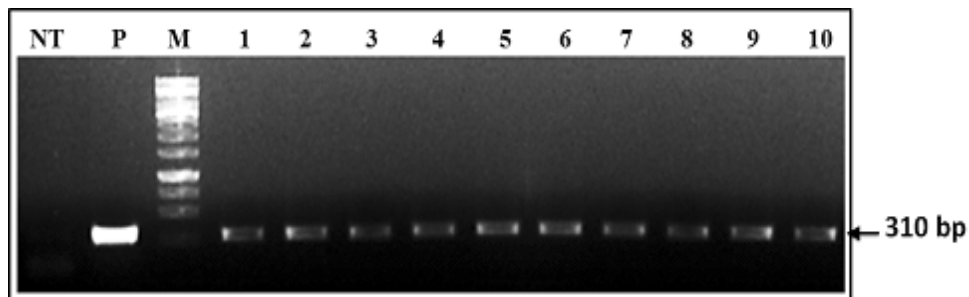


Figure 4. Screening of transgenics by genomic PCR analysis. Amplification of RGA2 intron (310bp) confirms integration of the transgene cassette in transgenic plants expressing RNAi vector constructs

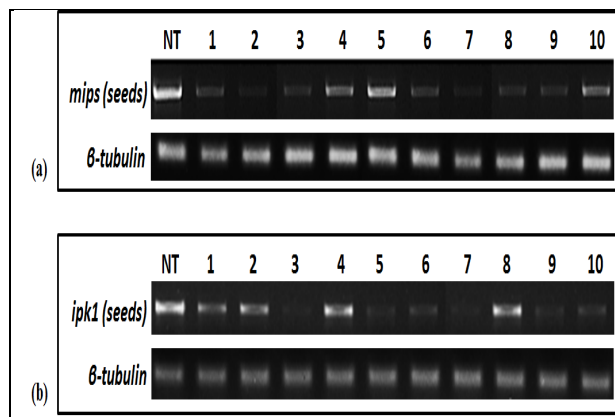


Figure 5. Semi-quantitative RT-PCR of (a) *mips* and (b) *ipk1* gene from the T_3 transgenic seeds. β -tubulin was used as the reference gene. (NT= Non-transgenic control, 1-10 = transgenics)

The transgenic also showed an increment of iron, not sequestered by phytic acid, which is attributed to be correlated to higher bioavailability of iron in rice seeds.

In future, we will also aim to silence other enzymes (viz, *ipk2*, *itp5/6k*) involved in the phytic acid biosynthesis (Suzuki et al 2007) and analyze its effect on the levels of phytate. In this context we have, already cloned aleurone tissue specific promoters from soybean, barley and wheat to mediate the tissue specific silencing of these enzymes in rice.

Carotenoid enriched rice

In order to develop carotenoid enriched rice grain, rice *psy1* and *crt1* genes were cloned under the control of endosperm specific *GluA2* promoter for *Agrobacterium* mediated transformation into local *indica* rice cultivar. Cloning and tissue specific expression of rice *psy3* gene from rice will be studied in recent future.

Nutritional quality during storage

Stability of nutritional quality of rice seed is very important during storage. It has been shown that co-oxidation of β -carotene takes place when lipoxygenase oxidizes polyunsaturated fatty acid. To develop transgenic rice with reduced lipoxygenase activity, RNAi technology is being used where the gene of interest is being controlled by seed specific promoter to down regulate lipoxygenase enzyme activity in seeds only. The *indica* rice cultivar, Pusa Sugandh 2 has been transformed with RNAi vector containing *lox3* gene. Molecular analyses like semi quantitative RT-PCR, Real time PCR and biochemical analysis confirmed the down regulated activities of *lipoxygenase* gene. The

effect of reduced lipoxygenase on nutritional quality deterioration due to storage is being analysed.

Breeding approaches

Introgression of high iron trait into local *indica* rice cultivar

The transgene mediated breeding strategy was already well-established phenomenon to improve the nutritional quality of the *indica* rice grains exhibiting the high carotenoid contents in the seeds (Datta et al 2007). In the present study, a similar kind of approach was employed to develop high iron local *indica* rice cultivars through the introgression of high iron trait (soybean *ferritin* gene). Soybean *ferritin* overexpressed FR-19-7 homozygous stable transgenic plants (developed by Vasconcelos et al 2003) were used for breeding purpose to introgress the high iron trait into local high yielding rice cultivar, Swarna. The *ferritin* positive F_1 plants were back-crossed with the parental Swarna twice and were grown under green house conditions. Successful introgression was confirmed through PCR with soybean *ferritin* gene specific primers (Fig. 6). From the atomic absorption spectroscopy results it is evident that iron and zinc contents were enhanced by ~ 2 -2.5 folds and ~ 1.2 -1.57 folds, respectively in the milled BC_2F_5 seeds due to the overexpression of soybean *ferritin* gene in the endosperm tissue (Fig. 7). Among the five hybridized progenies, FS3-3-8-1-1-1 line was considered as the most effective line due to its maximum iron accumulation properties (~ 17.0 $\mu\text{g/g}$ of seed dry weight). All the considered agronomical parameters of the hybridized plants and seeds exhibited similar kind of morphological traits with non-hybridized control Swarna plants.

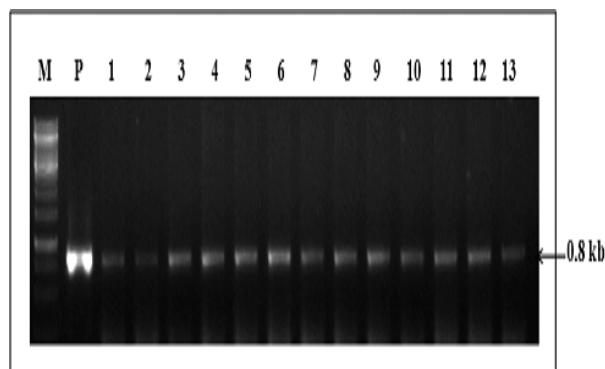


Figure 6. Gel picture showing PCR confirmation of *ferritin* gene in T_4 generation hybridized plants grown in green house (M= 1 Kb gene ruler, P= positive control, N= negative control, lane 1-17= plants positive for *ferritin* gene)

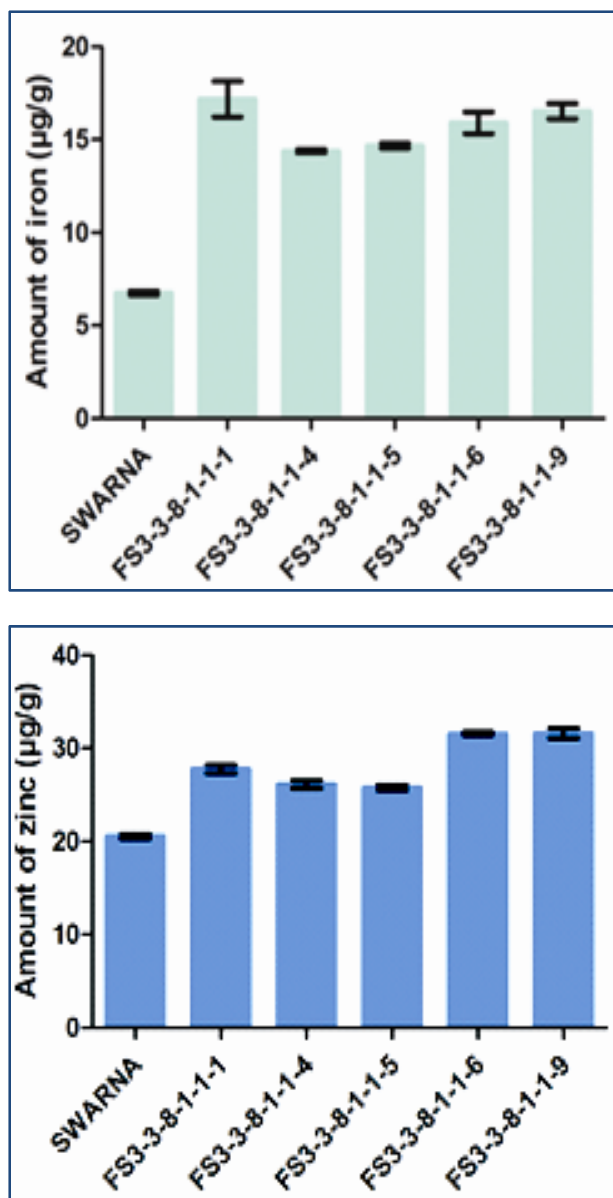


Figure 7. Amount of iron ($\mu\text{g/g}$) and zinc ($\mu\text{g/g}$) in milled F₅ hybridized seeds as compared to non-transgenic control (Swarna)

In future, low phytate trait will be introgressed into the *ferritin* overexpressor plants to develop the stable line of high iron enriched *indica* variety providing elevated amount of iron bioavailability

Discussion

In most of the developing countries, minerals and vitamin deficiencies in the cereal based diets may be the major reasons of several nutritional disorders. In the last 10-15 years, biotechnology has played a major role to develop nutrient enriched rice grain. Additionally, development of high iron cereal grains using different transporter mediated iron- loading mechanism also has a

significant impact on nutritional improvement (Masuda et al 2009; Zheng et al 2010). However, seed development, viability and germination may be seriously affected due to the presence of different heavy metals like Ni, Cd, etc. (Kranner and Colville 2011) during transport. Thus, without disturbing the metal transport mechanism, tissue specific overexpression of iron accumulators like ferritin and metabolic engineering like down regulation of phytic acid biosynthesis and manipulation of carotenoid biosynthesis pathway are very promising biotechnological approaches for micronutrient enrichment program.

The biotechnological approach of iron biofortification of rice through the tissue specific expression of rice ferritin itself is the preliminary step in the progress of cisgenic crop development. Effective overexpression of soybean ferritin in rice under the control of glutelinB1 and globulin1 promoters has already been reported (Qu et al 2005). However, overexpression of Osfer2 gene under *glutelinA2* promoter is a new approach for enhancement of iron nutrition in rice seeds. From RT-PCR data analysis of the transgenic seeds, it can be suggested that ferritin gene was successfully overexpressed in the endosperm tissues, which ultimately led to ~2.1 folds enhancement of iron in transgenic milled seeds (from AAS data). About 1.04-1.36 folds elevated levels of zinc accumulation in the transgenic seeds suggest the positive correlation between iron and zinc molecules, which was also similar to the development of soybean ferritin overexpressor seeds where accumulation of 2-3 folds of iron and ~1.6 folds of zinc was observed. Involvement of similar kind of transporter molecules like ZIP (*zrt/irt*-like proteins) or YSL (*yellow-stripe* like) may serve some positive relation between iron and zinc molecules (Waters and Sankaranan 2011).

Results from all the molecular and biochemical analysis, suggests that rice *ferritin* has appeared to be an impressive additive tool to increase iron nutrition in the grains.

Apart from the overexpression of rice *ferritin* gene, down-regulation of phytic acid biosynthesis is another useful innovative approach, which is attributed to increase the iron bioavailability in rice grain. In the present study we have developed two types of low phytate rice, one by silencing the myo-inositol-3 phosphate synthase enzyme (*mips*), which catalyzes the first step of phytic acid biosynthesis, and the other by disrupting the inositol 1,3,4,5,6-pentakisphosphate 2-kinase (*ipk1*) enzyme, involved in the last step of the biosynthesis. As revealed by molecular and biochemical analyses, the transgenic plants expressing both the genes

independently showed reduced level of phytate with a concomitant increase in inorganic phosphorus levels. However, the total phosphorus levels of both the transgenics and control appear similar, suggesting that the reduction in phytic acid has led to an increase in levels of available phosphate that is inorganic phosphorus. Further, the amount of iron as estimated by AAS in milled grains of low phytate transgenic rice strongly suggests that reduction of phytate has led to enhancement of iron in the consumable endosperm of transgenic rice seeds, which is correlated to the higher bioavailability of iron in seeds.

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